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Presence of a thapsigargin-sensitive calcium pump in *Trypanosoma evansi*: Immunological, physiological, molecular and structural evidences

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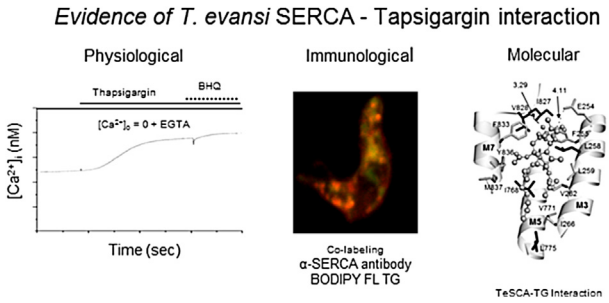
HIGHLIGHTS

- SERCA-like calcium pump from *Trypanosoma evansi* is sensitive to low concentrations of TG.
- TG induces calcium liberation from endoplasmic reticulum in *T. evansi*.
- *T. evansi* SERCA-like contains all ATPase P-type conserved domains from eukaryotes.
- *T. evansi* SERCA-like 3D model shares a high homology with SERCA1 crystal structure.
- Amino acid substitutions in *T. evansi* TG-SERCA cavity do not affect TG inhibition.

GRAPHICAL ABSTRACT



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ABSTRACT

In higher eukaryotes, the sarco-endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA) is characterized for its high sensitivity to low concentrations of thapsigargin (TG), a very specific inhibitor. In contrast, SERCA-like enzymes with different sensitivities to TG have been reported in trypanosomatids. Here, we characterized a SERCA-like enzyme from *Trypanosoma evansi* and evaluated its interaction with TG. Confocal fluorescence microscopy using BODIPY FL TG and specific anti-SERCA antibodies localized the *T. evansi* SERCA-like enzyme in the ER and confirmed its direct interaction with TG. Moreover, the use of either 1 μM TG or 25 μM 2′,5′-di (tert-butyl)-1,4-benzohydroquinone prevented the reuptake of Ca^{2+} and consequently produced a small increase in the parasite cytosolic calcium concentration in a calcium-free medium, which was released from the ER pool. A 3035 bp-sequence coding for a protein with an estimated molecular mass of 110.2 kDa was cloned from *T. evansi*. The corresponding gene product contained all the invariant residues and conserved motifs found in other P-type ATPases but lacked the calmodulin binding site. Modeling of the three-dimensional structure of the parasite enzyme revealed that the amino acid changes found in the TG-SERCA binding pocket do not compromise the interaction between

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the enzyme and the inhibitor. Therefore, we concluded that *T. evansi* possesses a SERCA-like protein that is inhibited by TG.

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1. Introduction

The Trypanosomatidae family encompasses a large number of unicellular organisms, many of which are causative agents of several tropical diseases that affect humans and animals. In South America, equine trypanosomosis, which is mainly caused by *Trypanosoma evansi*, is a seasonal and endemic parasitic disease that produces outbreaks of high mortality and great economical losses. The identification and characterization of novel parasite proteins as chemotherapeutic targets is a current trend for the development of new parasitic disease treatments. Some drugs used in the experimental treatment against trypanosomatids exert their action through the disruption of the parasite intracellular Ca^{2+} homeostasis, which usually results in cell death by apoptosis or necrosis (Benaim and Garcia, 2011). In general, Ca^{2+} is a major signaling molecule in eukaryotes including trypanosomatids, transmitting information within the cell and regulating numerous processes (Moreno and Doacampo, 2003). The Ca^{2+} cytosolic concentration is maintained at a nanomolar range (10–150 nM) by various Ca^{2+} transport systems that are located in intracellular organelles and the plasma membrane (Moreno and Doacampo, 2003). One of the major Ca^{2+} transporters or pumps is the sarcoplasmic/endoplasmic reticulum Ca^{2}-ATPase (SERCA), which is characterized by ATP stimulation and sodium orthovanadate inhibition (Vercesi et al., 1991; Moreno et al., 1992; Docampo et al., 1993).

In eukaryotic organisms, thapsigargin (TG), which is isolated from the roots of *Thapsia garganica* (Thastrup et al., 1990), and 2′,5′-di (tert-butyl)-1,4-benzohydroquinone (BHQ) (Oldershaw and Taylor, 1990) have become widely employed as pharmacological tools since they are specific inhibitors of SERCA. TG has a higher affinity for SERCA than BHQ, inhibiting the pump in an irreversible manner at nanomolar concentrations. In addition, TG possesses a high selectivity for SERCA given that no effect has been reported on other related Ca^{2}-ATPases, such as the plasma membrane Ca^{2+}-ATPase (PMCA) or the secretory pathway Ca^{2}-ATPase (SPCA), at these low concentrations (Michelangeli and East, 2011). The mechanism of inhibition by TG is highly understood. TG induces the release of Ca^{2+} from inositol-1,4,5-trisphosphate–sensitive intracellular stores, by locking the SERCA pump in a low Ca^{2+} affinity E2 state (Sagara et al., 1992). A crystal structure of TG-bound SERCA has been solved by x-ray diffraction studies (Toyoshima and Nomura, 2002). This structural report together with electron microscopy analyses showed that TG binds in the SERCA transmembrane core region near to the cytosolic surface, in a cavity delimited by the M3, M5 and M7 helices (Toyoshima and Nomura, 2002).

There is a controversy regarding the effect of TG on the store of Ca^{2+} in the endoplasmic reticulum (ER) of trypanosomatids. One report published the presence of a non-mitochondrial Ca^{2+} store in *Trypanosoma brucei* that was sensitive to 1 μM TG (Ruben and Akains, 1992). However, other reports have shown no effect of TG in both *T. brucei* (Vercesi et al., 1993) and *Trypanosoma cruzi* (Vercesi et al., 1991; Moreno et al., 1992) even at a concentration of 8 μM, and postulated that TG may have unspecific effects at high concentrations (4–20 μM) by collapsing the mitochondrial membrane potential (Docampo et al., 1993; Vercesi et al., 1993). Noland et al. (Nolan et al., 1994) suggested that the discrepancy in the effect of TG might be related to differences in the methodology employed by the various research groups. Yet, Mendoza et al. (Mendoza et al., 2004) demonstrated that 1 μM TG produced the release of intracellular Ca^{2+} before and after nigericin addition in *T. evansi* by spectrophotometrical Ca^{2+} measurements using whole cell populations, and intact and digitonin-permeabilized single cells. These results were in agreement with those obtained by Ruben and Akins (Ruben and Akains, 1992) for *T. brucei*.

T. brucei and *T. cruzi* SERCA have been cloned and overexpressed (Nolan et al., 1994; Furuya et al., 2001). Nolan et al. (Nolan et al., 1994) evaluated the sensitivity to TG of the recombinant SERCA from *T. brucei in vitro*, and found that the Ca^{2+}-ATPase activity of SERCA was sensitive to 1 μM of TG. However, Furuya et al. (Furuya et al., 2001) expressed the *T. cruzi* SERCA in yeast and found that this protein formed a Ca^{2+}-dependent ^{32}P-labeled phosphoprotein intermediate, which was blocked by ciclopiazonic acid but not by TG. Based on the residues that in SERCA are involved in TG binding and sensitivity (Nørregaard et al., 1994; Zong and Inesi, 1998), the differences observed in TG sensitivity by the *T. brucei* and *T. cruzi* proteins were associated with amino acids changes in the M3 and S3 fragments (Furuya et al., 2001). Other studies in trypanosomatids correlating the concentration of cytosolic Ca^{2+} with diverse cellular events, such as cell cycle control or invasion of mammalian cells, have shown that these processes are sensitive to 1 μM TG (Stojdl and Clarke, 1996; Yoshida et al., 2000; Neira et al., 2002). In the present study, we identified the SERCA-like Ca^{2+} pump from *T. evansi* (TeSCA) and evaluated its binding to TG. On the basis of the three-dimensional structure of the proposed TeSCA-TG complex obtained by molecular modeling, we suggest that TG and/or TG analogs might be used as a potential drug against *T. evansi*.

2. Materials and methods

2.1. T. evansi maintenance and purification

The Venezuelan *T. evansi* TEVA1 isolate (Desquesnes, 2004) was initially isolated from the blood of an infected horse from Apure State, Venezuela. Blood samples prepared in phosphate-buffered saline (PBS), pH 8, containing 1% glucose and 10% DMSO were preserved in liquid nitrogen. *T. evansi* parasites were expanded in healthy male rats (Sprague–Dawley, 300 g body weight) by intraperitoneal injection (100 μl) of cryo-preserved blood (~10^6 parasites/ml). Parasites were purified from whole blood of infected animals by ionic exchange chromatography (Lanham and Godfrey, 1970).

2.2. Western blot analyses

Aliquots from purified *T. evansi* lysates or the sub-cellular fraction enriched in purified rabbit muscle endoplasmic reticulum (ER) (Eletr and Inesi, 1972) containing 30 μg of total protein were separated by SDS-PAGE (Laemmli, 1970) and electrotransferred from gels to nitrocellulose sheets (0.45 mm pore size) (Towbin et al., 1979). Blots were blocked with 1% gelatin in TBS (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl) for 1 h. Then, incubated with a commercial polyclonal antibody specific for SERCA1/2/3 (H-300, Santa Cruz Biotechnology), diluted 1:500, and developed using

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